PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

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PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	11.10.2004
Applicant's or agent's file reference	**IMPORTANT NOTIFICATION**
E-1712/03	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP 03/05993	10.06.2003	07.06.2002

Applicant
IGEA S.R.L. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

   The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

   Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

   For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

   The applicant's attention is drawn to Article 33(5), which provides that the criteria of novelty, inventive step and industrial applicability described in Article 33(2) to (4) merely serve the purposes of international preliminary examination and that "any Contracting State may apply additional or different criteria for the purposes of deciding whether, in that State, the claimed inventions is patentable or not" (see also Article 27(5)). Such additional criteria may relate, for example, to exemptions from patentability, requirements for enabling disclosure, clarity and support for the claims.

Name and mailing address of the international preliminary examining authority:	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Evers, A Tel. +49 89 2399-7061

Form PCT/IPEA/416 (January 2004)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference E-1712/03	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP 03/05993	International filing date *(day/month/year)* 10.06.2003	Priority date *(day/month/year)* 07.06.2002
International Patent Classification (IPC) or both national classification and IPC C12N13/00		
Applicant IGEA S.R.L. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05.01.2004	11.10.2004
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Weijland, A Telephone No. +49 89 2399-7490

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 03/05993

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-16 as originally filed

Claims, Numbers

1-14 received on 06.07.2004 with letter of 05.07.2004

Drawings, Sheets

1/4-4/4 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations

   see separate sheet

The following documents (D) are referred to in this report; the numbering will be adhered to the rest of the procedure:

D1: WO-A-0181533
D2: WO-A-0107583
D3: WO-A-0107584
D4: WO-A-0107585

1. The amendments filed with the letter of 05.07.2004 meet the requirements of article 34(2)(b) PCT).

2. The subject matter of claims 1-14 is not disclosed in D1 to D4 or any other prior art document and is therefore novel (Article 33(2) PCT).

   D1 (abstract; page 2, lines 15-20) describes methods for electroporation including measuring the impedance, calculating an objective value of a signal on the basis of the predetermined impedance and supplying the substrate with a precalculated objective value Vo. The electrical field applied must be high enough to permeabilize the cell membrane, but not so high as to cause irreversible damage to the cell.

   D2 (abstract; Page 29, lines 13-24; claims 1-4) describes methods of controlled electroporation by passing a current across a medium comprising a cell part of a tissue. Electrical parameters may be current, voltage, impedance or a combination of current and voltage, which are used for determining the character of the cell and effect a membrane of the cell. An alternative method for detecting cells with damaged membranes would be to measure current-voltage relations across the cell, which produce immediate information. One possibility would be to use an electroporation chip for the process of electroporation. The measure of damage would be to measure the difference between an intact cell impedance and a damaged cell impedance as illustrated in Figure 8a and 8b. Thus, by modulating the amount of electrical current it is possible to obtain electroporation without significant damage to the cell thereby obtaining a reversible situation.

   D3 (abstract; page 18, lines 25 to 33; Figs 8a and 8b) describes controlled electroporation and mass transfer across cell membranes, by monitoring the electrical impedance, which detects the onset of electroporation. The amount of electrical current can be modulated to obtain electroporation without damaging the

cells.

D4 (abstract; page 28, lines 28 to 34; page 29, lines 1-5; Figs 8a and 8b) describes controlled electroporation and mass transfer across cell membranes, by monitoring the electrical impedance, which detects the onset of electroporation. The amount of electrical current can be modulated to obtain electroporation without damaging the cells.

3. Claims 1-14 do not appear to involve an inventive step (Article 33(3) PCT).

D2 is considered as the closest prior art document. Claim 1 differs from D2 in that D2 relates to a device which measures the electrical impedance to obtain a desired degree of electroporation.

The technical problem to be solved would reside in finding an alternative way to measure electroporation.

The skilled person, equipped with the knowledge of D2, would be motivated to arrive at the subject matter of claim 1, since the ratios voltage/current or current/voltage lead to the same technical effect, i.e. reflecting the permeability of the cells. The are just alternative numeric presentations of the identically measured parameters, i.e. voltage and current. Moreover, in D2 adjustments are carried out to facilitate analysis or to obtain a desired degree of electroporation ("controlled manner" according to claim 1), by measuring constantly the impedance. Moreover, it would be obvious to measure current and voltage as early ("initial portion" according to claim 1) as possible in the electroporation experiment, in order to adjust as fast as possible in order to obtain an optimal electroporation.

Therefore, claims 1-11 relating to an electroportion device and claims 12-14 relating to the use of such a device, do not involve an inventive step.

CLAIMS

1.- Electroporation device for the permeabilization of cells (C) contained in a substrate (12) comprising signal generating means (3) for generating a stimulating signal (S(t)) applied by means of electrodes (6,7) to the substrate (12) wherein an electric field (E(t)) permeabilizing the cells membranes is induced;

the device being **characterized by comprising:**

- means for measuring, calculating and monitoring (15,16,23) the instantaneous value of ~~a mathematical combination~~ the ratio (GT) of current (**ie**) flowing between said electrodes (6,7) and through the substrate (12) and the voltage (**Vp**) of the stimulating signal (S(t)) applied to the substrate (12) by means of said electrodes (6,7);

said device further comprising controlling means (100-170) for applying the stimulating signal in a controlled manner according to the waveform of an initial portion of the curve C_{GT} representing the ~~value of the mathematical combination~~ ratio (GT) in successive instants after the beginning of the application of the stimulating signal (S(t)).

2.- Device as claimed in claim 1, wherein said controlling means (100-170) comprise timing means (110) for applying said stimulating signal for a predetermined

period of time Td and analysing the initial portion of the waveform of curve C_{GT} to detect a minimum value of the curve C_{GT} within the interval t = 0 and t = Td.

3.- Device as claimed in claim 1 or 2, wherein said controlling means (100-170) calculate the slope of the waveform of curve C_{GT} after that a minimum in curve C_{GT} has been reached.

4.- Device as claimed in any of the preceding claims, wherein said controlling means (100-170) comprise hazard detecting means (120) determining the instantaneous gradient (dG) of said ~~mathematical combination~~ ratio (GT) after a minimum has been reached in said curve C_{GT};

said controlling means further comprise first comparing means (130) for comparing the calculated instantaneous gradient dG with at least a reference value (dG_{ref1}) and selecting correcting means (140,145) for performing an urgent correction to the stimulating signal S(t) in order to avoid lesions, damages or irreversible alterations in said substrate (12).

5.- Device as claimed in claim 4, wherein said correcting means (140,145) decreases the voltage of the stimulating signal S(t) in order to prevent deterioration in the cells (C).

6.- Device as claimed in any of the preceding claims, wherein said controlling means (100-170)

comprise slope determining means (150) calculating the average variation ΔG of said ~~mathematical combination~~ ratio (GT) in a time interval that is successive to the instant Tm wherein a minimum in the curve (C_{GT}) has been reached and that has a pre-determined time width;

said controlling means further comprising second comparing means (160) comparing the calculated average variation ΔG of said ~~mathematical combination~~ ratio (GT) with a reference interval of ΔG values.

7.- Device as claimed in claim 6, wherein said second comparing means (160) performs the following functions:

- if the calculated average variation ΔG of said ~~mathematical combination~~ ratio (GT) falls within the reference interval ($0 < \Delta G < \Delta G_{obb}$) continuing means (170) are selected;
- if the calculated average variation ΔG of said ~~mathematical combination~~ ratio (GT) falls outside the reference interval and it is smaller than both limits delimiting the interval ($\Delta G < 0 < \Delta G_{obb}$) adjusting means (180) are selected; and
- if the calculated average variation ΔG of said ~~mathematical combination~~ ratio (GT) falls outside the reference interval and it is greater than both limits delimiting the interval ($\Delta G > \Delta G_{obb} > 0$) correcting means (140) are selected.

8.- Device as claimed in claim 7, wherein said adjusting means (180) increase the voltage of the stimulating signal in order to increase the value of the electric field E(t) applied to the substrate (12); said adjusting means (180) subsequently selecting said means for calculating and monitoring (15,16,23) the ratio instantaneous value of the said ~~mathematical combination~~ (GT) and said controlling means.

9.- Device as claimed in claim 7, wherein said continuing means (170) increase the voltage of the stimulating signal to an objective voltage V_{opt} in order to increase the value of the electric field E(t) applied to the substrate (12) so that the value of said average variation ΔG tends to an expected value ΔG_{obb}.

10. - Device as claimed in any of the preceding claims, wherein said controlling means (100-170) detects (125) a minimum of said initial portion of said curve and determining (126) the time Tm at which the minimum is reached.

11.- Device as claimed in claim 10, wherein third comparing means (127) are provided to compare the detected time Tm with threshold values Ttmin and Ttmax;

said third comparing means (127) performing the following operations:

- if the detected Tm occurs before Ttmin (Tm < Ttmin) then correcting means (140) are selected;

- if the detected Tm occurs after Ttmax (Tm > Ttmax), then adjusting means (180) are selected; and

- - if the detected Tm occurs between Ttmin and Ttmax, then continuing means (170) are selected.

~~12. Device as claimed in any of the proceeding~~ claims, wherein said mathematical ~~combination~~ comprise the ratio GT ~~between~~ said current (ie) and said voltage ~~(Vp).~~

~~13. Electroporation device for the~~ permeabilization of cells (C) contained in a substrate (12) comprising signal generating means (3) for generating a stimulating signal (S(t)) applied by means of electrodes (6,7) to the substrate (12) wherein an electric field (E(t)) permeabilizing the cells membranes is induced;

the device being **characterized by comprising:**

- means for measuring, calculating and monitoring (15,16,23) the instantaneous value of the current **(ie)** flowing between said electrodes (6,7) and through the substrate (12);

said device further comprising controlling means (100-170) for applying the stimulating signal in a controlled manner according to the waveform of an initial portion of the curve representing the value of the current (ie) in successive instants after the

~~beginning of the application of the stimulating signal (S(t)).~~

12
~~14~~.- Use of a device as described in any of the preceding claims, to extract molecules from the living cells comprised in the substrate.

13
~~15~~.- Use of a device as described in any of the preceding claims, to introduce molecules into living cells.

14 13
~~16~~.- Use of the device as claimed in claim ~~15~~, wherein said molecules comprise one of the following:

- a DNA or a RNA molecule containing regulatory sequences and sequence coding for therapeutic genes or genes of interest for biomedical or biotechnological purposes;
- an oligonucleotide, (ribo- or deoxyribo-nucleotide, single or double strand, including the SiRNA), whether natural (phosphodiesters) or modified (inside the backbone of the oligonucleotide, such as phosphosulfates, or at the extremities, by addition of groups to protect the oligonucleotides from digestion of nucleases;
- a protein or peptide, whether natural or genetically or chemically modified, extracted from natural sources or obtained by synthesis, or a molecule simulating the structure of a

protein or peptide, whatever its structure;

- a cytotoxic agent, in particular, the antibiotic bleomycin or the cisplatinum;
- a penicillin; and

other pharmacological agents.